

Review



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We dedicate this paper to the late Craig Sandgren who made major contributions to our understanding of the Chrysophyceae and Syurophyceae.

Acquisition and metabolism of carbon in the Ochrophyta other than diatoms

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The acquisition and assimilation of inorganic C have been investigated in several of the 15 clades of the Ochrophyta other than diatoms, with biochemical, physiological and genomic data indicating significant mechanistic variation. Form ID Rubiscos in the Ochrophyta are characterized by a broad range of kinetics values. In spite of relatively high $K_{0.5CO_2}$ and low $CO_2 : O_2$ selectivity, diffusive entry of CO_2 occurs in the Chrysophyceae and Synurophyceae. Eustigmatophyceae and Phaeophyceae, on the contrary, have CO_2 concentrating mechanisms, usually involving the direct or indirect use of HCO_3^- . This variability is possibly due to the ecological contexts of the organism. In brown algae, C fixation generally takes place through a classical C3 metabolism, but there are some hints of the occurrence of C4 metabolism and low amplitude CAM in a few members of the Fucales. Genomic data show the presence of a number of potential C4 and CAM genes in Ochrophyta other than diatoms, but the other core functions of many of these genes give a very limited diagnostic value to their presence and are insufficient to conclude that C4 photosynthesis is present in these algae.

This article is part of the themed issue 'The peculiar carbon metabolism in diatoms'.

1. Introduction

Stramenopile eukaryotes comprise a diverse clade with a great range of nutritional modes [1] (table 1). Ochrophyta (i.e. Ochrista) is a diverse phylum of stramenopiles; most of the ochrophytan clades (16 in [5]) are photosynthetic or have photosynthetic members. The rest of the stramenopiles, the ecologically and economically very significant oomycetes and some others clades (a total of 8 in [2]), are all non-photosynthetic. The phylogenetic relations among these organisms are discussed by Baurain *et al.* [6], Brown & Sorhannus [7], Beakes *et al.* [2], Schmidt *et al.* [8] and Yang *et al.* [5].

The Bacillariophyceae is the best-investigated class of ochrophytes with respect to inorganic C acquisition and assimilation, showing the widespread occurrence of CO_2 concentrating mechanisms (CCMs) and of pyrenoids, energized transport of HCO_3^- and limited support for C₄ or C₄-like photosynthetic metabolism [9–12]. The emphasis on diatoms is reasonable in view of their phylogenetic and ecological diversity [13] and their contribution to global biogeochemical cycles (e.g. a marine diatom net primary productivity of about 20 Pg C per year [14]). However, there are 15 (possibly more) other ochrophyte classes with photosynthetic members [5–8,15]. The benthic marine macroalgal Phaeophyceae play a major role in primary productivity and as ecosystem engineers on rocky shores, especially in temperate and polar regions, with several species exceeding 10 m in length [16]. The Chrysophyceae and Synurophyceae are significant in the phytoplankton of acidic and often CO_2 -enriched freshwaters [3,17–19], the microalga *Nannochloropsis* (Eustigmatophyceae) is being assessed for its biotechnological potential [20–22] and members of the Pelagophyceae and

Table 1. Examples of modes of nutrition in the Ochrophyta. See Raven *et al.* [1] for definition of modes of nutrition.

| nutritional mode | example | references |
|--|--|------------|
| free-living osmoorganotrophy | most Saprolegniales, e.g. <i>Saprolegnia</i> (oomycetes) | [2] |
| symbiotic (all parasitic) ^a osmoorganotrophy | perenosporalean clades, e.g. <i>Phytophthora</i> ^b (oomycetes); bicoecids; <i>Cafeteria</i> | [2] |
| phagoorganotrophy | some Chrysophyceae, e.g. <i>Paraphysomonas</i> | [2] |
| photolithotrophy | examples: Bacillariophyceae, Eustigmatophyceae, Phaeophyceae, Synurophyceae | [3] |
| phagophotomixotrophy | many Chrysophyceae | [3,4] |

^aSymbiosis is used in the broad sense, including mutualism and parasitism.

^bMajor parasite of flowering plants.

Raphidophyceae can form harmful algal blooms [23]. Here we synthesize what is known about inorganic carbon acquisition and assimilation in these non-diatom ochrophytes for comparison with what is known for diatoms.

2. Mechanisms of inorganic C influx: CO₂ diffusion and biophysical CO₂ concentrating mechanisms

Diffusive CO₂ entry from the bulk phase to ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) is driven by a concentration difference produced by the photosynthetic consumption of CO₂ that produces a lower CO₂ concentration in the proximity of Rubisco active sites than in the bulk phase supplying the CO₂. Here the energization of the flux is indirect, coming from the use of absorbed photons for the reduction of NADP⁺ to NADPH and the phosphorylation of ADP to ATP; the NADPH and ATP are then used in CO₂ assimilation producing, in the first instance, carbohydrate. Under these circumstances, the CO₂ concentration and the CO₂:O₂ ratio at the Rubisco site typically allow significant Rubisco oxygenase activity and a corresponding operation of a photorespiratory C oxidation cycle that converts the 2-P-glycolate from Rubisco oxygenase into triose phosphate, with input of energy, directly or indirectly, from the thylakoid reactions of photosynthesis [24].

The functioning of CCMs, by definition, involves a higher steady state concentration of CO₂ at the site of Rubisco than occurs in the bulk medium. This transport of CO₂ against the free energy (concentration) gradient is energized independently of the energy input to the conversion of CO₂ to carbohydrate. This is the case for both the biophysical (energized, uphill transport of HCO₃⁻ or CO₂ or H⁺ across a cell membrane) and the biochemical (C4 photosynthesis or Crassulacean acid metabolism, CAM) CCMs. This parallel input of energy comes ultimately from the thylakoid reactions of photosynthesis, but sometimes indirectly via photosynthate production and its catabolism through glycolysis and the mitochondrial reactions [24]. Generally, despite the occurrence of CCMs, the CO₂:O₂ ratio at the Rubisco active site is not higher enough to prevent a small flux of organic C through Rubisco oxygenase and a photorespiratory C oxidation cycle that adds to the energy cost [25], as does the inevitable leakage of some of the CO₂ accumulated at the Rubisco site [24,26,27].

3. Inorganic C acquisition in Ochrophyta other than diatoms

(a) Introduction

There is substantial physiological evidence on the mechanism of inorganic carbon acquisition for a number of classes of the Ochrophyta, i.e. Chrysophyceae, Eustigmatophyceae, Phaeophyceae and Synurophyceae, with a little information available for a few other classes; genomic data are also available for the Eustigmatophyceae and the Phaeophyceae. We also discuss the occurrence of pyrenoids, showing that pyrenoids are not always required for stramenopile CCMs [9,28], and there can be pyrenoids but no obvious CCM activity [3].

A common rationale for the occurrence of diffusive CO₂ entry or of a CCM is the natural external CO₂ and O₂ concentrations and the kinetics and intracellular content of Rubisco. There is considerable variation in the kinetics, e.g. K_{0.5} for CO₂, K_{0.5} for O₂ and CO₂:O₂ selectivity, among the form ID Rubiscos in the Ochrophyta (table 2), as well as variations in the CO₂-saturated rate of CO₂ fixation per gram of Rubisco protein, with high CO₂-saturated specific reaction rates being generally associated with high K_{0.5} for CO₂ and a low CO₂:O₂ selectivity (= Srel or τ) [35]. However, significant variations of K_{0.5}(CO₂) and CO₂:O₂ selectivity may occur within the mechanistic possibilities suggested by Tcherkez *et al.* [35]; this is the case for diatoms [29], in which there is evidence of positive selection of Rubisco [36]. Diffusive CO₂ entry is more likely in organisms whose Rubiscos have a relatively low K_{0.5}(CO₂) and a high CO₂:O₂ selectivity; diffusive CO₂ entry would be particularly favoured if there is a CO₂ subsidy from the terrestrial catchment groundwater supply to rivers and some small lakes [37,38]. By contrast, Rubiscos with a high K_{0.5}(CO₂) and a low CO₂:O₂ selectivity combined with no CO₂ subsidy and approximately air-equilibrium CO₂ and O₂ concentrations would be compatible with the occurrence of a CCM. The data in table 2 show that this expectation is not always met, with diffusive entry of CO₂ in the Chrysophyceae and Synurophyceae, and CCMs in the Eustigmatophyceae and Phaeophyceae (see below). The occurrence of diffusive CO₂ entry in organisms with relatively high Rubisco K_{0.5}(CO₂) could be rationalized by the availability of sufficient energy, N and P (for the RNA needed for protein synthesis) to produce more Rubisco per cell and/or a large CO₂ subsidy.

Table 2. *In vitro* kinetics of ochrophytan Rubiscos. n.d., not determined.

| species | class | CCM | $K_{0.5}\text{CO}_2$ (mmol m^{-3}) | $K_{0.5}\text{O}_2$ (mmol m^{-3}) | CO_2/O_2 selectivity | references |
|-----------------------------|-------------------|-----|--|--|---|------------|
| 11 species | Bacillariophyceae | + | 23–68 | 413–2032 | 57–116 | [29] |
| <i>Nannochloopsis</i> sp. | Eustigmatophyceae | + | 7–10 | about 1000 | 27 | [30] |
| <i>Mallomonas papillosa</i> | Synurophyceae | – | 18.2 | n.d. | n.d. | [31] |
| <i>Synura petersenii</i> | Synurophyceae | – | 28.4 | n.d. | n.d. | [31] |
| <i>Synura uvella</i> | Synurophyceae | – | 41.8 | n.d. | n.d. | [31] |
| 4 species | Phaeophyceae | + | 12–43 | n.d. | n.d. | [32] |
| <i>Olisthodiscus luteus</i> | Raphidophyceae | ? | 45–59 | 692 | 101 | [9,33,34] |

(b) Chrysophyceae and Synurophyceae

The sister classes Chrysophyceae and Synurophyceae both have diffusive CO_2 transport from the bulk medium to Rubisco. Most of the data relate to the Synurophyceae [3,17,31,39–42], with some for the Chrysophyceae [3,41], which, as indicated in table 1, are frequently phagomixotrophic [43].

The evidence on inorganic C entry includes pH drift. Through this method, it was possible to show that the photosynthetic activity of these algae can increase the pH and deplete CO_2 to values attributable to a diffusive CO_2 entry. More direct estimates of relatively high CO_2 compensation concentrations from net photosynthetic rates as a function of inorganic C concentration, as well as estimates of photorespiration, support the view of inorganic carbon acquisition in Chrysophyceae and Synurophyceae as mostly due to CO_2 diffusive entry driven by consumption of CO_2 in the chloroplasts. For the two cases investigated (one Chrysophyceae and one Synurophyceae), there is no external carbonic anhydrase activity [3]. Vegetative cells of the Synurophyceae and cysts of the Chrysophyceae and Synurophyceae have extraprotoplasmic silicification, in common with several other clades of the Ochrophyta, especially the Bacillariophyceae and Bolidophyceae [44,45]. Among other roles of silicification is that as a pH buffer facilitating H^+ movement related to extracellular carbonic anhydrase activity [46]; this cannot be the case in the Chrysophyceae and Synurophyceae with no extracellular carbonic anhydrase. Despite the lack of CCMs, pyrenoids occur in some Chrysophyceae (*Chromulina*, but not *Ochromonas*) [3,9] and Synurophyceae (e.g. *Synura*) [3,47].

For the Synurophyceae, there are also comparisons of the *in vitro* $K_{0.5}$ of Rubisco and *in vivo* photosynthesis. The form ID Rubiscos of the Synurophyceae have $K_{0.5}$ values (mmol m^{-3}) *in vitro* of 18.2 (*Mallomonas papulosa*), 28.4 (*Synura petersenii*) and 41.8 (*Synura uvella*) [31]. The photosynthetic $K_{0.5}(\text{CO}_2)$ are 92.0–440.5 mol m^{-3} for *M. papillosa* (varying with the buffer used to maintain the pH at 7.0), 40.4–43.7 mol m^{-3} (varying with pH 6–7) for *S. petersenii* and 44.9–209 mol m^{-3} for *S. uvella* (varying with pH 6–7) [31]. The *in vivo* photosynthetic $K_{0.5}$ values can be accommodated by the *in vitro* Rubisco $K_{0.5}$ with diffusive CO_2 entry, granted a Rubisco content that gives a V_{max} for Rubisco equal to the V_{max} for *in vivo* photosynthesis, and allowing for the necessary decrease in CO_2 concentration along the diffusion pathway from the bulk medium to Rubisco [41]. The Rubisco assays used unpurified

cell extracts [31], so the CO_2 -saturated Rubisco specific reaction rate cannot be calculated.

(c) Eustigmatophyceae

Eustigmatophyceae appear to all have a CCM, when cells are grown in culture media in equilibrium with the present atmosphere. The freshwater *Eustigmatos vischeri* and *Vischeria stellata* can take up both CO_2 and HCO_3^- [48–50], while the soil-dwelling *Monodus subterraneus* can only use CO_2 [48,49]. Most of the data are available for the marine *Nannochloropsis* spp. (*N. gaditana*, *N. oceanica* and *N. salina*) and *Monallantus* sp., where mass spectrometric and other evidence show HCO_3^- influx powered by mitochondrial respiration, with significant simultaneous efflux of CO_2 [48,49,51–58]. Merrett *et al.* [53] showed that HCO_3^- influx was inhibited by DIDS (4'4'-diisothiocyanostilbene-2,2'-disulfonic acid), an inhibitor of anion exchangers, and by the absence of Cl^- , consistent with the occurrence of a $\text{HCO}_3^- : \text{Cl}^-$ antiport of unknown stoichiometry. The energized HCO_3^- influx is downregulated when cells are grown at high CO_2 [58], consistent with decreased CCM expression at high CO_2 when diffusive CO_2 entry can provide as high a CO_2 concentration at the active site of Rubisco as does the CCM at lower external CO_2 concentrations. However, Merrett *et al.* [53] were unable to demonstrate a higher internal than external inorganic C concentration using silicone oil centrifugation, just as Huertas *et al.* [48,49] were unable to do from CO_2 efflux kinetics just after cessation of illumination. Huertas *et al.* [48] suggest that the intracellular pool occupies a small fraction of the cell volume and/or that *Nannochloropsis* Rubisco had, like red algal Rubiscos, a low $K_{0.5}$ for CO_2 . This latter suggestion was verified by Tchernov *et al.* [30], who found a $K_{0.5}$ for CO_2 of extracted *Nannochloropsis* sp. Rubisco of 7–10 mol m^{-3} , the lowest values in table 2. This notwithstanding, the need for a CCM exists in *Nannochloropsis* due to a very low $\text{CO}_2 : \text{O}_2$ selectivity of 27 [30] and incomplete suppression of Rubisco oxygenase activity *in vivo* [59]. Expressing the photosynthetic $K_{0.5}$ for HCO_3^- in terms of CO_2 gives $K_{0.5}$ values of 0.27 mmol m^{-3} for *Monallantus* sp. and 0.63 mmol m^{-3} for *Nannochloropsis gaditana* [52], i.e. an order of magnitude higher affinity than for the Rubisco of *Nannochloropsis* sp. [30], indicating the involvement of a CCM. Furthermore, the occurrence of a net CO_2 efflux in the light with all inorganic C entering as HCO_3^- and high affinities for inorganic C, a close approximation to inorganic C saturation at seawater inorganic C concentration, a low (zero)

and O₂-insensitive CO₂ compensation point, a high ability to photosynthesize at high pH and no inhibition of photosynthesis by 21 kPa O₂ relative to 2 kPa O₂ means that there has to be a higher internal than external CO₂ concentration. Pyrenoids are apparently universal in the Eustigmatophyceae (but see [60]), in parallel with the occurrence of CCMs [61,62].

The genomic data show probable HCO₃⁻ transporters of the anion exchanger family at the plasmalemma (Nga00165.01) and the chloroplast envelope (Nga06584) of *Nannochloropsis gaditana* CCMP526 [21]; see www.nannochloropsis.org/gene/Naga_10007g30 and www.nannochloropsis.org/gene/Naga_10007g124. The *Nannochloropsis oceanica* CCMP1779 genome has two genes that resemble the LCIA 1595 in *Chlamydomonas* [63,64]: one is a chloroplast envelope carrier protein (CCP), which is induced by low CO₂ (CCMP1779_7325-mRNA-1); the other is the LCIA protein (CCMP1779_6536-mRNA-1), which belongs to the formate/nitrite transporter family, has an unknown location and is also induced under low CO₂ [22]. Using the same strain of *N. oceanica*, Poliner *et al.* [65] found two SL4 HCO₃⁻ transporters. The SLC4 family from metazoans catalyses a variety of HCO₃⁻ transport processes, i.e. 1 HCO₃⁻ : 1 Cl⁻ exchanger, 1 HCO₃⁻ : 1 Na⁺ cotransporter, 2 HCO₃⁻ : 1 Na⁺ cotransporter, 3 HCO₃⁻ : 1 Na⁺ cotransporter and a (2 HCO₃⁻ + 1 Na⁺) : 1 Cl⁻ exchanger [66]. Of these, only the 1 HCO₃⁻ : 1 Na⁺ cotransporter could lead to inorganic carbon accumulation in the cytosol, granted the probable gradients of Na⁺ and Cl⁻ across the plasmalemma [67,68]. SLC4 HCO₃⁻ transporters are also known from the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* and the brown alga *Ectocarpus siliculosus* [65]. The occurrence of SL4 HCO₃⁻ transporters is not diagnostic of CCMs since they are found in the C₃ terrestrial flowering plant *Arabidopsis thaliana* [65] and metazoans [66]. Li *et al.* [69] found two low CO₂-induced putative formate/nitrite transporters in the plastid envelope of *N. oceanica* IMET1.

(d) Phaeophyceae

The largest ochristan algae occur in the Phaeophyceae [16]. The final pH and corresponding equilibrium CO₂ concentration achieved in pH drift experiments with Phaeophyceae show HCO₃⁻ use or, less likely, energized accumulation of CO₂ in almost all cases [70–78], with the proviso that the final pH may be restricted by pH *per se* rather than by limitations on the removal of inorganic C from seawater [41,79]. Similar conclusions arise from the meta-analysis by Stepien [80] of δ¹³C natural abundance of organic C of Phaeophyceae collected from their natural habitats, with the proviso that the ¹³CO₂ : ¹²CO₂ discrimination of the isolated Rubisco of the only ochristan tested, the diatom *Skeletonema costatum* [81], differs from that assumed by Stepien [80] and the authors that she cites.

Of the intertidal and subtidal Phaeophyceae from the northeast Atlantic examined by Johnston & Raven [82–84], Surif & Raven [85,86] and Johnston [32], the intertidal species (Fucales) show high affinities for inorganic C, a close approximation to inorganic C saturation at seawater inorganic C concentration, a low (zero) and O₂-insensitive CO₂ compensation concentration, a high ability to photosynthesize at high pH and no inhibition of photosynthesis by 21 kPa O₂ relative to 2 kPa O₂. By contrast, the subtidal Laminariales have a lower affinity for inorganic C, lack of inorganic C saturation at seawater inorganic C concentration, a higher and

O₂-sensitive CO₂ compensation concentration, a smaller ability to photosynthesize at high pH and some inhibition of photosynthesis by 21 kPa O₂ relative to 2 kPa O₂. These characteristics indicate the occurrence of a CCM in all species; the CCM of the Laminariales appears to be less developed in terms of inorganic C affinity and O₂ insensitivity of photosynthesis than that of the Fucales. The work of Johnston & Raven [82–84], Surif & Raven [85,86] and Johnston [32] involved experiments under submersed and under emersed conditions with (where identical treatments were used) closely similar results, although the external availability of both HCO₃⁻ and CO₂ in submersed experiments and external availability of only CO₂ in emersed experiments leaves unanswered questions about the mechanism of inorganic C entry to cells.

Why, in ecological and evolutionary terms, is there this difference between intertidal and subtidal brown algae in the northeast Atlantic? We need to look at other possible determinants of zonation, such as tolerance of desiccation, especially for high-intertidal algae, which can be emersed for long periods during neap tides [16]. One would expect that desiccation tolerance would be important for high-intertidal algae, especially when emersed for long periods at spring tides. Surif & Raven [86] suggest that a high affinity for inorganic C of intertidal brown algae in the NE Atlantic increases the photosynthetic C gain per emersion period. However, there are several intertidal (some very high intertidal) red algae lacking CCMs [41], e.g. *Bostrychia arbuscula* (formerly *Stictosiphonia arbuscula*) in New Zealand in the high-intertidal zone occupied by *Pelvetia canaliculata*, a fucoid with a CCM, on NE Atlantic coasts [16,87].

The extent of suppression of Rubisco oxygenase and hence of photorespiration by CCMs of Phaeophyceae has been investigated by Surif & Raven [86] using a comparison of photosynthetic rates as a function of CO₂ from 50 to 950 μmol mol⁻¹ total gas with O₂ at 20 or 210 mmol mol⁻¹ total gas. The photosynthetic rates of the five intertidal species of the Fucales (Fucales) were unaffected by the different O₂ levels [84] while those of the normally submerged Laminariales and the normally submerged member of the Cystoseiraceae (Fucales) were lower in 210 than in 20 mmol mol⁻¹ total gas at all the CO₂ levels tested [86]. The O₂ inhibition of the normally submerged algae differs from that of typical C₃ physiology plants by the absence of CO₂-O₂ competition.

Other data on O₂ inhibition of photosynthesis in the Phaeophyceae [88–92] show increased glycine and serine production (as markers for the photorespiratory carbon oxidation cycle) as a fraction of total inorganic C assimilated with increasing O₂ [90]. The presence of the photorespiratory carbon oxidation cycle in the Phaeophyceae has been shown by enzyme assays [93,94].

Much of the information on other members of the Phaeophyceae show characteristics more like those of normally submerged algae than the intertidal algae studied by Johnston & Raven [82–84], Surif & Raven [85,86] and Johnston [32]. Examples of the inorganic C affinity and O₂ sensitivity of photosynthesis by other members of the Phaeophyceae are provided by Black *et al.* [88], Downton *et al.* [89], Burris [90], Dromgoole [91,92] and Raven *et al.* [71–73]: see review by Raven & Hurd [95].

The general assumption is that the CCM is based on the direct or indirect use of external HCO₃⁻ rather than on active influx of CO₂. HCO₃⁻ use could involve direct HCO₃⁻ transport using an anion transport protein in the

plasmalemma that is usually detected in macroalgae by inhibition of photosynthesis by DIDS and/or SITS (4'-acetamido-4'-isothiocyanostilbene-2,2-disulfonic acid). Such transport is known for the gametophyte, but not for the sporophyte phase of the kelp *Undaria pinnatifida* [96] and for the sporophyte phase (gametophyte not examined) of the giant kelp *Macrocystis pyrifera* over a wide range of pH values [28]. No inhibition of photosynthesis by DIDS was found for the members of the Desmarestiales, Fucales, Laminariales, Sphacelariales or Scytosiphonales studied by Larsson & Axelsson [97] or Zou & Gao [74]. Evidence of HCO_3^- entry in *E. siliculosus* (Ectocarpales) comes from experiments at an external pH of 9.5, where CO_2 is less than 0.23 mmol m^{-3} [98]. In these experiments, the photosynthetic rate at pH 9.5 was still 30% of that at pH 7.9 and was insensitive to buffering of the medium with 50 mol m^{-3} of either cationic or anionic pH buffers, or to inhibition of external carbonic anhydrase, thus ruling out the mechanism of HCO_3^- use discussed in the two following paragraphs [98]. HCO_3^- entry could account for part of the photosynthesis of *E. siliculosus* at an external pH of 7.9–8.2 ([98]; cf. [99]). At an external pH of 8 in photosynthesis-saturating red light, *E. siliculosus* accumulates $4\text{--}5 \text{ mol m}^{-3}$ inorganic C, i.e. about twice the external concentration; this internal pool is decreased to about 2 mol m^{-3} when blue light is added, with photosynthetic consumption of the released inorganic C [100].

The alternative mechanism is based on localized acidification of the surface of the organism using energized H^+ efflux, although the diffusion boundary layer of photosynthesizing cells is at a higher pH than the bulk medium [101]. The acid zones have an equilibrium $\text{CO}_2\text{:HCO}_3^-$ ratio that increases by an order of magnitude with each pH unit decrease, and there is also an order of magnitude increase in the rate of uncatalysed HCO_3^- conversion to CO_2 [101]. The higher concentration of CO_2 at the cell surface has two fates; one is to leak back to the bulk phase, the other is a biologically useful diffusion into the cell to Rubisco. This mechanism is commonly supplemented by the presence of extracellular carbonic anhydrase [102]. The generation of surface acid areas to facilitate CO_2 diffusion towards Rubisco is well characterized in freshwater Charales and some submerged freshwater flowering plants, where each acid zone has an area of more than 1 mm^2 . Price & Badger [103] showed that this mechanism of HCO_3^- use is inhibited by higher concentrations ($20\text{--}50 \text{ mol m}^{-3}$) of pH buffers of an appropriate pK_a . Such attempts that have been made to identify acid zones on the surface of the Phaeophyceae during photosynthesis have not identified areas in the square millimetres [95], although the techniques used were not suited to identifying smaller acid areas. Transient surface acidification occurring with blue light added to photosynthesis-saturating red light in a range of brown algae [104] shows that surface acidification can occur during photosynthesis in the Phaeophyceae.

In the absence of direct measurements of sustained localized surface acidification, the occurrence of a CCM of the kind proposed by Walker *et al.* [101] has been suggested (see discussion above) on the basis of inhibition of photosynthesis by pH buffers and by inhibitors of extracellular carbonic anhydrases. Sometimes the absence of inhibition by the HCO_3^- transporter inhibitors DIDS and SITS has also been used as negative evidence for acid zone-dependent CCMs. Support for a Walker *et al.* style [101] CCM by the use of purported inhibitors of H^+ efflux catalysed by plasmalemma

H^+ -ATPase is equivocal because the H^+ gradient generated could also be used to indirectly energize a $\text{Na}^+\text{--HCO}_3^-$ symport influx of HCO_3^- . With these provisos, the mechanism that occurs in the Laminariales other than gametophytes of *U. pinnatifida* and sporophytes of *M. pyrifera* is consistent with a mechanism based on that described by Walker *et al.* [101] for *Chara* ([105–107]; see also [108,109]), as is some evidence for this mechanism in a member of the Scytosiphonales [74], but not for a member of the Fucales [77]).

Despite the occurrence of CCMs, most brown algae lack pyrenoids although these structures have evolved independently in several clades of the Phaeophyceae [9,28,78,110,111].

The genome of *Ectocarpus siliculosus* contains a putative $\text{Na}^+\text{--HCO}_3^-$ transporter targeted to the chloroplast, and a putative $\text{Cl}^-\text{--HCO}_3^-$ transporter with no clear targeting [112,113]. Nakajima *et al.* [11] and Poliner *et al.* [65] found two SLC4 HCO_3^- transporters in the genome of *E. siliculosus*; other Ochrophyta have two (in the eustigmatophycean *N. oceanica* and the centric diatom *T. pseudonana*) and four (in the pennate diatom *P. tricornutum*) SLC4 genes. The *P. tricornutum* PtSLC4-2 is targeted to the plasmalemma, is inhibited by the anion transporter inhibitor DIDS and is Na^+ -dependent. Ye *et al.* [114] report the genome sequence of the kelp (Laminariales) *Saccharina japonica*; the analysis by Bi & Zhou [115], however, does not mention HCO_3^- transporters or H^+ -ATPases, but does show a range of carbonic anhydrases. Genomic data indicate the occurrence of many of the enzymes of the photorespiratory carbon oxidation cycle [112].

(e) Raphidophyceae and Tribophyceae

Little is known of the inorganic C acquisition by the Raphidophyceae, other than Rubisco kinetics [33,116]. The *in vitro* $\text{K}_{0.5}$ for CO_2 of Rubisco in the marine *Olisthodiscus luteus* is 45 mmol m^{-3} at 23°C (table 2); for the marine *Heterosigma carterae*, the $\text{K}_{0.5}$ for inorganic C dependence of the rate of photosynthesis *in vivo*, expressed in terms of CO_2 , is 3 mmol m^{-3} at 16°C [117]. This difference suggests that a CCM is operative in the algae of this class. The pH drift work of Nimer *et al.* [118] indicated low photosynthetic rates for the marine *Heterosigma akashiwo*; little can be deduced from these data about the mode of inorganic C acquisition. Pyrenoids seem to be universal in the Raphidophyceae [119]. For the Tribophyceae (i.e. Xanthophyceae), the only data are those of Beardall and Entwisle [120] on the terrestrial–freshwater *Botrydiopsis intercedens*. In this species, internal inorganic C accumulation occurs to a greater extent than it would for diffusive CO_2 entry, in accord with the pH gradient; this is suggestive of the operation of a CCM. Several members of the Tribophyceae have pyrenoids [9] though there seem to be no data on *B. intercedens*. There are no data on inorganic C acquisition by the sister clade to the diatoms, the Bolidiophyceae, now known to have the Parmales as the silicified cyst phase [121].

(f) Pyrenoids and CO_2 concentrating mechanisms

As indicated above for individual taxa, pyrenoids seem to be essential features of some CCMs based on active transport across membranes [9,113]. However, pyrenoids occur in some species of the Chrysophyceae and Synurophyceae, classes that uniformly lack CCMs, while the Phaeophyceae, apparently with CCMs in all taxa, generally lack pyrenoids, with several independent origins of pyrenoids. In the Eustigmatophyceae, all members investigated have CCMs and

pyrenoids. In the Synchronophyceae, a condensed pyrenoid is often present in the chloroplast stroma [8], although there is no evidence on the occurrence of CCMs in this class. This is also the case for the Pelagophyceae, in which *Aureaumbra* (at least) has pyrenoids [122].

4. Inorganic C assimilation in Ochrophyta other than diatoms

(a) Introduction

All photosynthetic, eukaryotic C assimilation pathways have the photosynthetic carbon reduction cycle (PCRC, or Calvin–Benson cycle), with Rubisco as the carboxylase, at their core. Raven *et al.* [25] discussed alternative inorganic C assimilation pathways found in some autotrophic Archaea and Bacteria, and energetic, inorganic C affinity and O₂ damage as reasons why many of them are not appropriate for functioning in the present atmosphere. In some organisms, the PCRC is downstream of diffusive entry, in others it is downstream of a ‘biophysical’ CCM based on active transport of HCO₃⁻, CO₂ or H⁺ to produce C₃ photosynthetic biochemistry. In some oxygenic photosynthetic organisms, the PCRC is downstream of a C₃–C₄ cycle in C₄ photosynthesis; this is a ‘biochemical’ CCM, although it occurs downstream of a ‘biophysical’ CCM in some aquatic flowering plants. In C₄ photosynthesis, there is close temporal coupling of the C₃–C₄ and PCRC cycles with small pool sizes of the C₄ and C₃ intermediates. Another upstream C₃–C₄ cycle contributes to crassulacean acid metabolism (CAM); here there is a temporal lag of about 12 h between the scotophase acidification with CO₂ fixation into malate, which is stored as malic acid in vacuoles, and deacidification in the photophase, where CO₂ is released by decarboxylation of malic acid, and refixed by Rubisco and the PCRC, with the C₃ moiety from malate decarboxylation stored until the next scotophase as mono- or polysaccharide.

The biochemistry of autotrophic CO₂ assimilation was determined in pre-molecular biology by two main methods. One was short-time (seconds) exposure of the organism to ¹⁴CO₂ (terrestrial organisms) or ¹⁴C inorganic C (aquatic organisms) in the light, followed by rapidly killing the organism and quantification of the water- or ethanol-soluble organic compounds labelled with ¹⁴C. C₃ biochemistry is characterized by 3-phosphoglycerate (PGA) as the initial labelled compound, followed by PCRC sugar phosphates and then compounds derived from the PCRC; labelling of C₄ dicarboxylic acids in anaplerotic processes is only a few percent of that through the PCRC. C₄ biochemistry has C₄ dicarboxylic acids as the initial labelled products, followed by PGA and other PCRC compounds; a few seconds labelling (pulse) followed by a change back to unlabelled inorganic C (chase) shows a decrease in label in PGA and an increase in label in C₄ dicarboxylic acids. This was the method used in the 1950s and 1960s to establish the C₃ pathway of autotrophic inorganic C assimilation in green microalgae (*Chlorella*, *Scenedesmus*) and the terrestrial flowering plant *Hordeum*, and the C₄ pathway in the terrestrial flowering plants *Saccharum* and *Zea*. The other, much less decisive, method is determination of the activity of carboxylase enzymes in cell extracts; a high Rubisco : PEPC (phosphoenolpyruvate carboxylase-oxygenase) activity ratio has been taken to indicate C₃ biochemistry, and a low ratio (less than 1) is suggestive of C₄ biochemistry.

(b) Phaeophyceae

Both the kinetics of labelling of organic compounds after addition of ¹⁴C inorganic C and the *in vitro* activity of carboxylases methods have been applied to the Phaeophyceae [32,123–128]. The kinetics of the ¹⁴C-inorganic C labelling method shows C₃ biochemistry in a range of brown algae [32,123,127–129]. In the brown algae examined by Küppers & Kremer [130], Kremer [129] and Hillrichs & Schmid [127] there is significant labelling of C₄ acids (aspartate, malate) in the light, and the time course of the label position within aspartate shows that the PEP comes from photosynthetic 3-PGA, with slower incorporation of ¹⁴C-inorganic C into the β-carboxyl of aspartate by phosphoenolpyruvate carboxylase (PEPCK) or, more likely, PEPC. The ratio of ¹⁴C-inorganic C labelling of organic C (initially mainly into aspartate and malate) by carboxylation of PEP to that carried out by Rubisco is lowest in mature tissue and highest in growing tissue, consistent with an anaplerotic role of PEP carboxylation, although the rates may be higher than the anaplerotic requirement [129,130]. ¹⁴C-inorganic C labelling of organic C (mainly aspartate and malate) occurs in the dark at a rate higher than that of green and red marine macroalgae and is also probably higher than the anaplerotic requirement [129]. As indicated below, in some fucoids, a part of this dark ¹⁴C-inorganic C assimilation can be attributed to low amplitude CAM.

Possibly related to the labelling of C₄ acids mentioned above is the finding of a ‘buffer system’ taking up (at high pH in the dark) and releasing (at normal seawater pH in the light) inorganic C, as indicated by stimulation of O₂ production in the light in North Atlantic intertidal members of the Fucales (species of *Ascophyllum*, *Fucus* and *Pelvetia*; all Fucaceae) [131,132]. This ‘buffer system’ is associated with H⁺ exchange between seawater and the algae, is not found in other subtidal brown algae examined, i.e. *Halidrys*, Fucales (Cystosieraceae), *Desmarestia* (Desmarestiales) and *Laminaria* (Laminariales), and is paralleled by a particular spatial organization of organelles in the outer cell layer (meristoderm) of the thallus [131,132]. A more widespread phenomenon among brown algae is the stimulation of photosynthesis by additional blue light to already saturating red light [133,134], which involves release in blue light of at least some of the inorganic C from an intracellular pool accumulated in red light [100]. The blue light effect was not found in the only diatom examined, *P. tricorutum* [133]. Possibly related to the two preceding sets of data, but probably not, is the interesting finding in the work of Johnston [32] on ¹⁴C inorganic C pulse-chase incorporation in the North Atlantic intertidal *Ascophyllum nodosum* of a continued increase in label incorporation in organic compounds in the chase period, in algae collected in July 1998 but not in January 1988. After a 5 s pulse in July 1988, the increase in label of organic C seems to be saturated at the longest time period tested (300 s) at about eight times the organic C label at the end of the pulse period. This requires a very substantial accumulation of an acid labile pool of either inorganic C or of an acid-labile organic C compound in the 5 s pulse, with incorporation into a range of acid-stable compounds in the chase period. Kawamitsu and Boyer [135] used the North Atlantic intertidal *Fucus vesiculosus* and showed that, after exposure to light in seawater, photosynthetic O₂ production continued for a decreasing rate over 2 h in CO₂ free air. A small fraction of the C store on which this O₂ production was based was inorganic C; most of it was organic, presumably (an

organic acid(s). Unlike CAM, and the work of Axelsson *et al.* [131,132], this C store was not filled during the scotophase [135], although there is evidence of minor CAM inorganic C assimilation in several furoid brown algae including *F. vesiculosus* [136]. Axelsson *et al.* [131,132] comment that their 'photosynthetic buffer' may be related to the very low CAM activity found in the species showing the 'photosynthetic buffer'.

The enzyme analyses showed significant activity of PEPCK, but higher activities of Rubisco [32,123,126]. PEPCK activity could not be detected in these investigations, including that of Busch & Schmid [126] on *E. siliculosus*, although genomic evidence indicates that PEPCK occurs in this alga [112].

The most recent tool for distinguishing between C₃ and C₄ (and CAM) photosynthetic biochemistry is genomics and transcriptomics. However, the presence of genes encoding enzymes used in C₄ (and CAM) photosynthesis such as PEPCK, PEPCK, PPDk (phosphate pyruvate dikinase, sometimes considered for diagnostic C₄ photosynthesis), MDH (malate dehydrogenase), NADP-ME (NADP malic enzyme) and NAD-ME (NAD malic enzyme), is not of itself sufficient to show that C₄ photosynthesis occurs. One reason is that the enzymes have other functions and occur in photosynthetic organisms known to use C₃ biochemistry [137,138]. Another is that the enzyme might not be expressed in an intracellular location compatible with C₄ photosynthesis [139]. Bi & Zhou [115] have produced a generalized diagram (their fig. 1) including C₄ photosynthesis and list genes [114] compatible with the PEPCK C₄ mechanism (granted appropriate intracellular localization of the enzymes), but this is not evidence demonstrating that C₄ photosynthesis occurs. The conclusion of Gravot *et al.* [112] is that there is not clear evidence from genome analysis as to the occurrence of C₄ photosynthesis in the model brown alga *E. siliculosus*.

The other inorganic C assimilation pathway found in the Phaeophyceae is very low amplitude CAM in some members of the Fucales, but not in other orders of the brown algae [112,136].

(c) Raphidophyceae and Eustigmatophyceae

For other photosynthetic Ochrophyta, Descolas-Gros & Oriol [140] found activity of PEPCK but not PEPCK in *H. akashiwo* (Raphidophyceae). However, PEPCK was found in the proteome of another raphidophycean, *Aureococcus anophagefferens* [141].

Genomic data on *N. gaditana* (Eustigmatophyceae) shows PEPCK, MDH (NAD(P) malic dehydrogenase) and PPDk in the cytosol, and NAD(P)-ME in the chloroplast, that could be part of C₄ photosynthetic biochemistry, as well as pyruvate carboxylase (PC) in the mitochondria (fig. 6 of [21]).

Genomic data on *N. oceanica* CCMP1779 (Eustigmatophyceae) show the presence of PEPCK, MDH, PPDk and NAD(P)-ME, but the intracellular location is not indicated (table S10 of [22]). Vieler *et al.* [22] (their table S10) show that expression of probable inorganic C transporters is increased at low CO₂ concentrations, but make no comment about the effects of CO₂ on expression of the enzymes that could be involved in C₄ photosynthesis. Li *et al.* [69] used transcriptomic data on *N. oceanica* IMET1 to predict the location of enzymes that could be part of a C₄ photosynthesis and found that enzyme location is not entirely as required for an effective C₄ pathway.

Table 3. Summary of mechanisms of inorganic carbon acquisition and assimilation among classes in the Ochrophyta. No data are available for the Aureanophyceae, Bolidophyceae, Chrysochromophyceae, Dictyochophyceae, Pelagophyceae, Phaeothamniophyceae, Pinguicophyceae, Schizodactylophyceae and Synchronophyceae. See the main text for details.

| class | Rubisco kinetics | diffusive CO ₂ entry | CCM | C ₃ biochemistry | C ₄ biochemistry | CAM | phagotrophy |
|-------------------|------------------|---------------------------------|--------------|-----------------------------|--|-------------------------------|-------------------------------|
| Bacillariophyceae | yes | no | yes | yes | C ₃ -C ₄ in some? | no | no |
| Chrysophyceae | no data | yes | no | assumed C ₃ | no data | unlikely | no |
| Eustigmatophyceae | yes | no | yes | assumed C ₃ | ? | unlikely | no |
| Phaeophyceae | yes | no | yes | yes | some production of C ₄ acids in the light | low amplitude in some species | low amplitude in some species |
| Raphidophyceae | yes | probably not | probably yes | no data | no data | unlikely | no |
| Synurophyceae | yes | yes | no | no data | no data | unlikely | no |
| Tribophyceae | no data | no | yes | no data | no data | unlikely | no |

5. Conclusion

We only have a significant body of information on inorganic C acquisition and assimilation for four non-diatom classes of Ochrophyta, i.e. the Chrysophyceae, Eustigmatophyceae, Phaeophyceae and Synurophyceae, with some information on the Raphidophyceae and Tribophyceae (table 3). Diffusive CO₂ entry from the bulk medium to Rubisco occurs in the Chrysophyceae and Synurophyceae, while the Eustigmatophyceae and Phaeophyceae have CCMs involving influx of HCO₃⁻ and/or CO₂ (table 3).

The Phaeophyceae is the only class for which there is biochemical evidence on the pathway of inorganic C assimilation, showing that there is predominantly C₃ biochemistry, but with occasional elements of C₄ biochemistry and low-amplitude CAM in some members of the Fucales.

The energization of CCMs involves active influx of HCO₃⁻ or active efflux of H⁺ at the plasmalemma, with little or no role for C₄ biochemistry, and no net inorganic C entry in the dark in the few brown algae with low amplitude CAM. The distribution of pyrenoids does not completely parallel that of CCMs based on active transport across membranes.

Molecular genetic investigation has not provided definitive evidence as to the occurrence of C₄ photosynthesis and has indicated possible membrane transporters involved in CCMs. Some members of the Fucaceae, and *Ectocarpus*, have

incompletely explained inorganic C reservoirs, as inorganic C and/or as (presumably) carboxylate C that can be readily converted to inorganic C.

Returning to the topic of the other papers in this thematic issue, i.e. C metabolism in the Bacillariophyceae, the other classes of Ochrophyta for which information is available show a greater diversity of mechanisms of inorganic C acquisition and biochemistry of autotrophic CO₂ assimilation. The occurrence of CCMs is less widespread among other ochrophytes than in diatoms, as is the correlation between the occurrence of CCMs and the presence of pyrenoids. While C₄ or C₄-like photosynthetic metabolism is a possibility in both diatoms and other ochrophytes, no diatom is known to have the low-amplitude CAM found in some fucal brown algae.

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